Suppression by Apoptotic Cells Defines Tumor Necrosis Factor-Mediated Induction of Glomerular Mesangial Cell Apoptosis by Activated Macrophages

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Suppression by Apoptotic Cells Defines Tumor Necrosis Factor-Mediated Induction of Glomerular Mesangial Cell Apoptosis by Activated Macrophages

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Activated macrophages (Mϕ) isolated from inflamed glomeruli or generated by interferon-γ and lipopolysaccharide treatment *in vitro* induce glomerular mesangial cell apoptosis by hitherto incompletely understood mechanisms. In this report we demonstrate that nitric oxide-independent killing of co-cultured mesangial cells by interferon-γ/lipopolysaccharide-activated Mϕ is suppressed by binding/ingestion of apoptotic cells and is mediated by tumor necrosis factor (TNF). Thus, soluble TNF receptor-1 significantly inhibited induction of mesangial cell apoptosis by 1) rodent Mϕ in the presence of nitric oxide synthase inhibitors or 2) human Mϕ, both situations in which nitric oxide release was minimal. Furthermore, murine TNF knockout Mϕ were completely unable to induce mesangial cell apoptosis in the presence of nitric oxide synthase inhibitors. We conclude that TNF-restricted Mϕ-directed apoptosis of glomerular mesangial cells can be down-regulated by Mϕ binding/ingestion of apoptotic cells, suggesting a new mechanism for negative feedback regulation of Mϕ controls on resident cell number at inflamed sites. *(Am J Pathol 2001, 159:1397–1404)*

Proliferation of resident cells is a prominent feature of inflammatory responses. In glomerular inflammation there is typically an increase in number of resident glomerular mesangial cells that adopt a myofibroblast-like phenotype, lay down excess abnormal matrix, and thereby threaten progression to scarring.[1] However, in self-limited nephritis excess mesangial cells are deleted by apoptosis and the glomerular cell complement returns to normal.[2,3] Until recently the mechanisms mediating deletion of myofibroblast-like mesangial cells have been obscure. However, we showed that activated macrophages (Mϕ) can direct apoptosis of such cells,[4] mesangial cell killing being mediated by nitric oxide (in a rodent cell system) and another unknown factor. Because inflammatory Mϕ can delete neutrophils[5] and a range of tumor cells[6–8] by tumor necrosis factor (TNF)-mediated mechanisms, there was a strong possibility that activated Mϕ might use TNF to induce mesangial cell apoptosis, particularly in human cell systems in which Mϕ production of nitric oxide is notoriously difficult to detect.[9]

Furthermore, the demonstration that activated/inflammatory Mϕ can kill resident glomerular cells immediately begs the question as to how the killing capacity of Mϕ might be regulated. Importantly, work from Reiter and colleagues[10] demonstrated that the capacity of rodent bone marrow-derived Mϕ stimulated with interferon (IFN)-γ and lipopolysaccharide (LPS) to induce tumor cell apoptosis was diminished to ~30% of control by ingestion of apoptotic cells. However, although this was associated with a modest reduction in nitric oxide production to ~75% of control, the Mϕ killing mechanism(s) suppressed by ingestion of apoptotic cells was/were not characterized further.[10]

In this study we set out to determine whether activated Mϕ induction of glomerular mesangial cell apoptosis was suppressed by Mϕ ingestion of apoptotic cells and to determine which Mϕ mechanisms for triggering apoptosis in neighboring mesangial cells were subject to such control.

Materials and Methods

Materials

Media and fetal calf serum (FCS) were purchased from Life Technologies (Paisley, UK). Tissue culture plastic was from Falcon (Becton Dickinson, Mountainview, CA) and Costar (Cambridge, MA) as stated in the text. Cyto-

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kines were purchased from R&D Systems (Minneapolis, MN) and all other reagents from Sigma (St. Louis, MO) unless otherwise stated.

Cell Isolation and Preparation

Human mesangial cells were obtained from the cortex of fresh nephrectomy specimens. Mesangial cells were purified from outgrowths of whole, purified glomeruli and passaged according to standard techniques in full Dulbecco's modified Eagle's medium (DMEM)/F12 (with 10% FCS, and supplemented with penicillin and streptomycin (Life Technologies).[2] Rat mesangial cells were derived from outgrowths of whole glomeruli as described.[4] They were passaged according to standard techniques in full DMEM/F12, and were used between passage 6 and 14. Rodent macrophages were derived from bone marrow taken from the femur of Wistar rats or from murine strains (TNF$\alpha/\beta-/-$) and wild-type littermate controls (C57BL6 $\times$ 129sv). In the TNFR1-Fc blockade studies, they were all C57BL6). Marrow was prepared and cultured in Teflon bags with murine M-CSF as previously described.[4] Cells were used after 7 to 10 days, then were plated into wells 16 hours before experimentation to ensure adequate adhesion and correct cell number. Human macrophages were derived from peripheral blood monocytes. Briefly, peripheral blood mononuclear cells were obtained from the buffy coat of fresh peripheral blood from healthy donors. Red cells were removed by dextran sedimentation and granulocytes were separated by centrifugation through a discontinuous Percoll gradient.[11] The monocytes were then purified using the MACS monocyte cell isolation system (Miltenyi Biotech, Cologne, Germany). Purity of monocytes (>95%) was established by flow cytometric characteristics. Monocytes were then cultured in Teflon wells (2×10^6/ml) in Iscove's-modified DMEM with 10% autologous serum. Medium was changed on day 2 and day 4. Differentiation was confirmed by cytology on cytospins and CD14 immunofluorescence (not shown).

Generation of Apoptotic Cells

Rat mesangial cells were induced into apoptosis by ultraviolet irradiation. Subconfluent monolayers in T75 flasks (Costar) were exposed to UV irradiation (312 nm, 8 W, 3 minutes) followed by incubation for 16 hours. Nonadherent (apoptotic) cells were removed by agitation and collected in the supernatant. After centrifugation, apoptotic cells were further purified by washing with phosphate-buffered saline (PBS) 1$\times$ and centrifugation (190 $\times$ *g,* 5 minutes). Apoptosis was confirmed by histology, selective uptake of Hoechst 33342 (1 μg/ml), but exclusion of propidium iodide (PI) (1 μg/ml). Typically, <10% of cells were PI-positive and <5% did not exclude trypan blue (0.2%).

Mouse thymocytes were prepared from 20 g C57/BL6 mice by pressing the thymus through a 50-μm sieve. The single cell supernatant was resuspended (2×10^6 cells/ml) in RPMI supplemented with glutamine and 2-mercaptoethanol in addition to 10% FCS and antibiotics. Cells were either exposed to a 5-minute burst of UV irradiation (312 nm, 8 W) followed by 2.5 hours of culture, or dexamethasone (1 μmol/L) followed by culture for 6 hours. Typically >50% of induced cells were apoptotic (Annexin V binding; Boehringer Mannheim. Mannheim, Germany) and permeable to Hoescht 33342 (1 μg/ml)) whereas <5% of those were positive for the uptake of PI (flow cytometric analysis, data not shown).

Co-Culture of Mϕ and Mesangial Cells

For a detailed description, see our earlier work.[4] Matured rodent bone marrow-derived macrophages were plated in 96-well plates initially at a density to cover 60 to 70% of the well surface; typically this required 2×10^4cells per well. Rat mesangial cells were prelabeled with CellTracker Green 5-chloromethylfluorescein diacetate CMFDA (Molecular Probes, Eugene, OR): cell cultures, 70 to 80% confluent, were washed with medium lacking serum and then incubated for 1 hour in serum-free medium containing CMFDA at 5 ng/ml. Cells were washed in medium containing 10% FCS to remove any unbound CMFDA, then trypsinized and added to cultured rodent Mϕ in a 1.5 Mϕ:1.0 mesangial cell ratio, previously shown to be optimal for demonstration of macrophage-directed mesangial cell apoptosis.[4] Experiments were performed in DMEM/F12 medium containing 10% FCS. Once cells had become adherent, typically 2 to 4 hours, wells were washed to remove nonadherent cells. In our earlier work[4]mixing unlabeled and labeled cells showed no evidence of transfer of CMFDA from labeled to unlabeled cells. Rodent co-cultures were activated with IFN-γ (100 U/ml) plus LPS (1 μg/ml).

In some experiments, unlabeled apoptotic cells (1×10^5 per well of a 96-well plate) were added to the established co-culture and to the control wells of mesangial cells alone, at the same time as activating cytokines. As a control for apoptotic cells, aliquots of 10-μm diameter sterile latex beads (Polysciences, UK) were added to adjacent co-culture. When thymocytes were used as apoptotic cells, aliquots were added to co-culture for 6 hours, then noningested cells gently washed away, before activation. In separate wells containing Mϕ alone, apoptotic cells or latex beads were also incubated for 6 hours, then washed off, and wells were fixed for quantification of percentage Mϕ that had phagocytosed apoptotic cells. This was assessed by phase contrast microscopy. Mϕ (34 $\pm$ 5.6%) phagocytosed at least one apoptotic thymocyte and 30 $\pm$ 6.7% phagocytosed apoptotic mesangial cells. Mϕ (67 $\pm$ 8.4%) ingested latex beads. By contrast only 1 $\pm$ 1% of Mϕ phagocytosed the population of live thymocytes in this assay.

For human co-culture, human monocyte-derived Mϕ were plated at 2×10^4 per well as above. Cycling human mesangial cells were primed with IFN-γ (500 U/ml) for 24 hours, then prelabeled with CMFDA as described for rat mesangial cells. Once washed and trypsinized, they were added to wells in a 2:1 ratio (because of the larger size of human mesangial cells). After 4 hours, wells were

washed and replaced with full DMEM/F12. Human co-cultures were activated with human IFN-γ (Peprotech) (500 U/ml) and LPS (1 μg/ml).

Assessment of Mesangial Cell Apoptosis in Co-Culture

For a detailed description, see our earlier work.[4] At the end of co-culture experiments, wells were either fixed with formaldehyde (4% final concentration) and stored for 48 hours at 4°C to allow firm adhesion of apoptotic cells to the plate, or they were assessed live by fluorescence microscopy after the addition of Hoechst 33342 (1 μg/ml) and PI (1 μg/ml). For assessment of live cells, green rounded-up cells were scored as apoptotic if they also were positive for Hoechst uptake, but excluded PI. For assessment of fixed (and permeabilized) cells by morphology, plates were first counterstained with PI (1 μg/ml) and Hoechst (1 μg/ml) in PBS for 5 minutes (which stains both Mϕ and mesangial cells). After discarding the stain, wells were covered with a fluorescent mountant. Apoptotic mesangial cells were easily discernible by green fluorescence and characteristic morphology. For both live and fixed wells, apoptosis of mesangial cells was assessed blindly and five fields per well were randomly chosen without observer bias. Each experiment was performed in triplicate. Previous studies have shown this method to give similar results to flow cytometric assays.[4] However, the microscopical assay is reproducibly more sensitive because apoptotic mesangial cells tend to disintegrate during centrifugation once they have been fixed. Note that when exogenous apoptotic cells were added these were not labeled with fluorescent dyes, enabling confident identification of apoptosis in the previously healthy target mesangial cells.

Assays of TNF-α and Nitric Oxide

Culture supernatants (free from phenol red) were harvested, clarified by centrifugation (4000 × *g,* 5 minutes) then stored at −20°C. After complete thawing, 50 μl of each sample was assayed by Quantikine mouse TNF-α enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instructions. A standard curve with absorbencies from 0.1 to 1.2 was achieved on each occasion. Samples (50 μl) were assayed for nitrite by mixing with an equal volume of the Griess reagent as previously described[6] and measuring absorbency at 540 nm and comparing with a sodium nitrite standard curve.

Chimeric Soluble Death Receptors

Fusion proteins were constructed using cDNAs for the extracellular domains of human receptors fused with the Fc portion of human IgG_1. Proteins were expressed in insect cells infected with recombinant baculoviruses. Protein secreted into the culture supernatant was then purified by protein A-Sepharose column affinity. The protein was stored at −20°C in Hanks' salt solution.[12]

Knockout Mice

TNF-α/TNF-β double-knockout mice were generated by inserting a targeting vector between exon 1 and 2 of the murine TNF-β gene and the middle of exon 4 of the adjacent murine TNF-α gene of chromosome 17 of GS1 mouse embryonic stem cells. Mutant embryonic stem cells were selected and injected into C57/BL6 blastocysts.[13]

Statistics

All experiments were performed on at least four separate occasions using at least four animals. The data were expressed as mean values with the SE of mean. Paired data were compared using the *t*-test and multiple comparisons using analysis of variance.

Results

Ingestion of Apoptotic Cells by Activated Rodent Mϕ Selectively Suppresses Nitric Oxide-Independent Induction of Mesangial Cell Apoptosis

In our previous studies of IFN-γ/LPS-activated rodent bone marrow-derived Mϕ killing of rodent mesangial cells primed with IFN-γ (Figure 1) we demonstrated that at least half of the mesangial cell killing was independent of Mϕ-derived nitric oxide, being unaffected by inhibitors of inducible nitric oxide synthase (iNOS) such as L-NMMA at 500 μmol/L.[4] We also observed that the vast majority of mesangial cells induced into apoptosis by activated Mϕ were not ingested,[4] presumably because of kinetic and spatial restraints in a two-dimensional culture system in which IFN-γ-primed mesangial cells were added above a sparse monolayer of Mϕ before the co-culture was activated by addition of IFN-γ and LPS.

Therefore, to test whether ingestion of apoptotic cells diminished Mϕ capacity to trigger apoptosis in mesangial cells, an established co-culture of IFN-γ-primed rat mesangial cells with rat Mϕ was exposed to a fivefold excess of mesangial cells induced into apoptosis by UV irradiation; the apoptotic cells were administered at the same time as activation of the co-culture with IFN-γ and LPS. Under standard conditions, there was no significant effect of apoptotic cells on Mϕ induction of mesangial cell apoptosis. However, in the presence of 200 μmol/L L-NMMA, apoptotic mesangial cells exerted a dramatic inhibitory effect on Mϕ induction of mesangial cell apoptosis (Figure 2). Addition of the same number (1×10^5 per well) of 10-μm latex beads, as a control phagocytic particle, had no inhibitory effect whether L-NMMA was present or not (Figure 3). Furthermore, suppression of nitric oxide-independent triggering of apoptosis was observed with rodent thymocytes induced into apoptosis by UV or dexamethasone, but not when freshly isolated non-apoptotic thymocytes were used as controls (Table 1). Finally, similar results were observed whether apoptotic

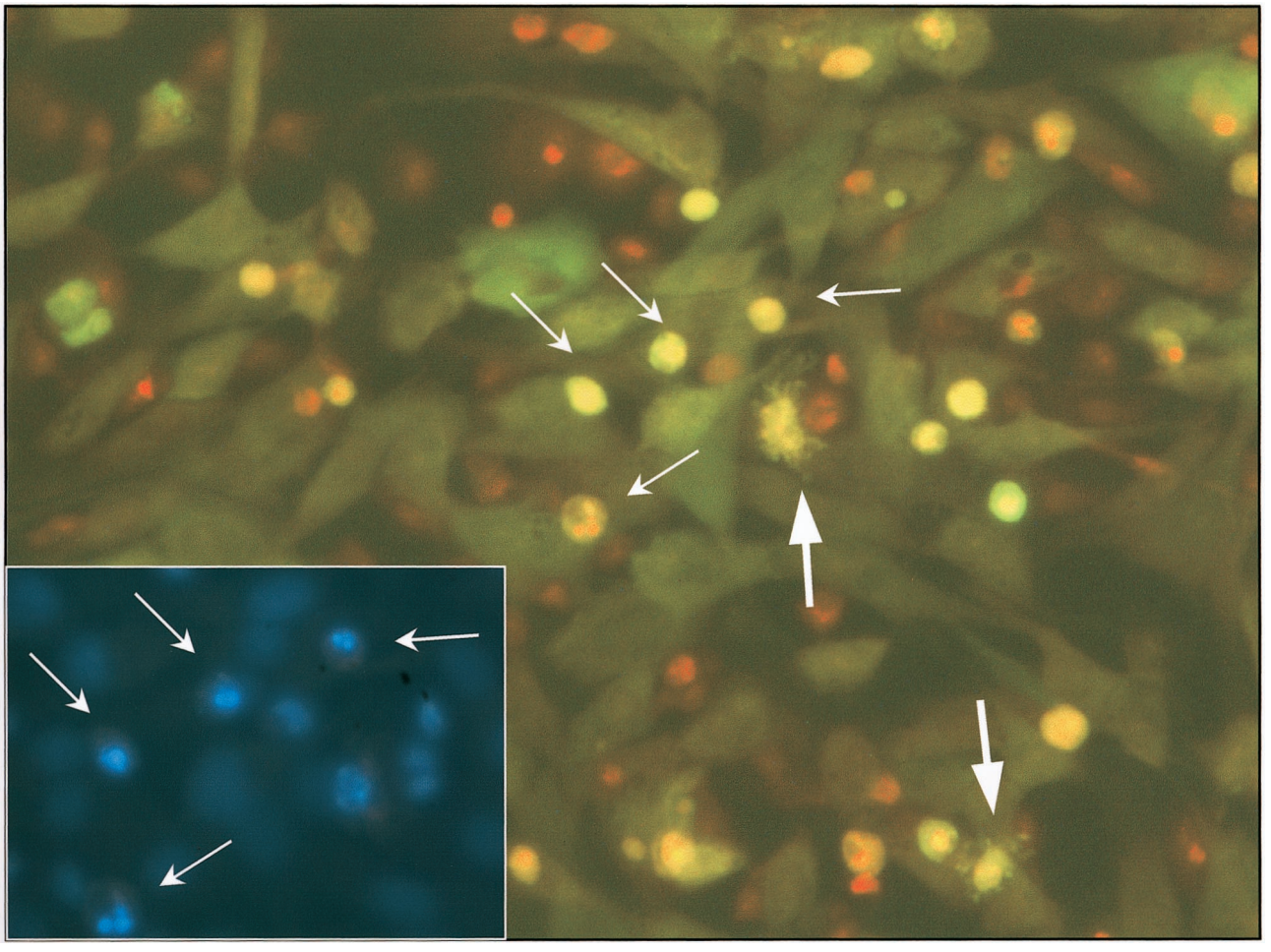

Figure 1. Fluorescent micrograph (original magnification, ×320) showing activated co-culture of CMFDA green-labeled mesangial cells with Mϕ. The co-culture was established according to Materials and Methods and activated with IFN-γ (100 U/ml) plus LPS (1 μg/ml) after 4 hours of exposure to preformed unlabeled apoptotic mesangial cells. At 24 hours, the co-culture was fixed and later counterstained with PI (1 μg/ml). Apoptotic green mesangial cells are clearly seen with red/orange pyknotic nuclear material and are readily distinguished from Mϕ (**arrows**). Before fixation, co-culture was exposed to Hoechst 33342 (1 μg/ml) for confirmation of apoptosis of the green mesangial cells (see **inset**). Note **inset** field and **arrow**-marked apoptotic cells correspond to central field of main figure. Note also two apoptotic mesangial cells showing cytoplasmic blebbing (**large arrows**).

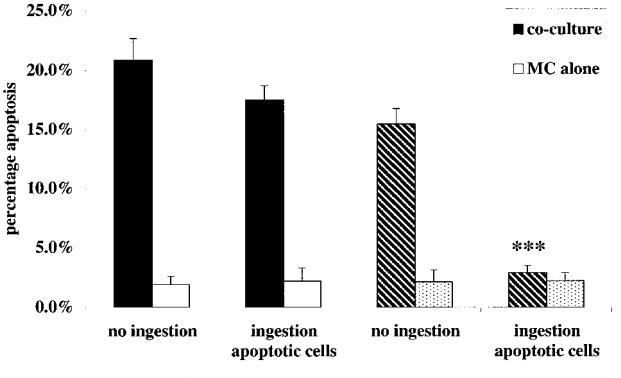

Figure 2. Induction of rodent mesangial cell apoptosis by activated Mϕ is blocked by simultaneous phagocytosis of apoptotic mesangial cells when iNOS inhibitors are present. Established co-cultures of rodent Mϕ and IFN-γ primed, CMFDA-labeled, mesangial cells, or mesangial cells growing alone (as control), were activated with IFN-γ (100 U/ml) and LPS (1 μg/ml). Simultaneously to activation, a fivefold excess of unlabeled apoptotic rodent mesangial cells was added (1×10^5 cells per well). In addition, to some wells (**hatched bars**), L-NMMA (200 μmol/L) was added at the same time as activation. After 24 hours of incubation, induction of CMFDA-labeled mesangial cells was scored in all wells. ***, $P < 0.001$ *versus* no ingestion ($n = 5$).

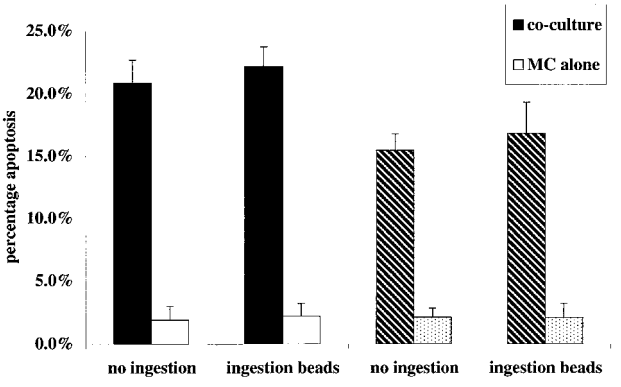

Figure 3. Induction of rodent mesangial cell apoptosis by activated Mϕ is not blocked by simultaneous phagocytosis of latex beads when iNOS inhibitors are present. Both established co-cultures of rodent Mϕ- and IFN-γ-primed, CMFDA-labeled, mesangial cells, and wells of mesangial cells alone were activated with IFN-γ (100 U/ml) and LPS (1 μg/ml). Simultaneously a fivefold excess of unlabeled sterile 10-μm latex beads was added (1×10^5 cells per well). In addition, to some wells (**hatched bars**), L-NMMA (200 μmol/L) was added. After 24 hours of incubation, induction of CMFDA-labeled mesangial cells was scored ($n = 5$).

Table 1. The Effect of Co-Culture with Apoptotic Cells upon Murine Mϕ Induction of Apoptosis in IFN-γ Primed Mesangial Cells at 24 Hours in the Presence of Nitric Oxide Synthesis Inhibitor

Cell type	% Mesangial cell apoptosis	
	No added cells	Cells added
Apoptotic mesangial cells (UV)	10.7 ± 2.1	3.3 ± 1.0*
Apoptotic thymocytes (UV)	16.1 ± 1.4	8.2 ± 0.1†
Apoptotic thymocytes (Dex)	16.1 ± 1.4	8.3 ± 0.5†
Nonapoptotic thymocytes	16.1 ± 1.8	14.2 ± 1.3

UV, Ultraviolet; Dex, dexamethasone.

Rodent co-culture was established as described in Materials and Methods. Apoptotic cells were incubated with the co-culture for 6 hours. Mϕ were activated with IFN-γ plus LPS in the presence of L-NMMA 200 μmol/L after washing away noningested cells. Note that control cultures of mesangial cells alone exposed to apoptotic cells followed by activating cytokines underwent no greater than 3.42 ± 0.2% apoptosis.

*$P < 0.01$ compared with no added cells.

†$P < 0.001$ compared with no added cells.

cells were added simultaneously with activation of co-culture, as above, or if apoptotic cells were administered to co-culture before activation (Table 1). This indicates that the suppressive effect was mediated by effects of apoptotic cells on the Mϕ most likely consequent on ingestion of apoptotic cells, but not excluding an effect of binding alone.

Blockade of TNFR1 Also Inhibits Nitric Oxide-Independent Macrophage Induction of Mesangial Cell Apoptosis

Exposure of activated monocytes/macrophages to apoptotic cells exerts a well-established inhibitory effect on secretion of TNF.[14,15] It therefore seemed especially likely that nitric oxide-independent induction of IFN-γ-primed mesangial cell apoptosis by activated Mϕ, which we had found to be inhibited by apoptotic cells (Figure 2), was also mediated by Mϕ-derived TNF.

To seek a role for TNF in Mϕ directed killing of mesangial cells, we examined the effect of soluble chimeric death receptors (fused with the Fc portion of human IgG_1) on activated rat macrophage killing of primed rat mesangial cells in the presence of inhibitors of nitric oxide synthesis (in these experiments the selective iNOS inhibitor L-NIL at 30 μmol/L, as validated in our earlier work[4]). A role for Mϕ-derived FasL had already been ruled out in rodent co-culture[4] by using bone marrow Mϕ from *gld/gld* mice (that lack active FasL) and therefore soluble inhibitors of Fas-FasL interaction were not used. Induction of rat mesangial cell apoptosis in activated co-culture was assessed at 24 hours. TNFR1-Fc (10 μg/ml) was able to reduce significantly the killing capacity of Mϕ in co-culture (Figure 4) whereas lymphotoxin β-receptor fusion protein (LTβR-Fc[16]) and herpesvirus entry mediator fusion protein (HVEM-Fc[17,18]) had no effect. At higher concentrations, TNFR1-Fc (50 μg/ml) was able to increase further the reduction in killing capacity (14.3 ± 3.3% rat mesangial cell apoptosis in activated co-culture

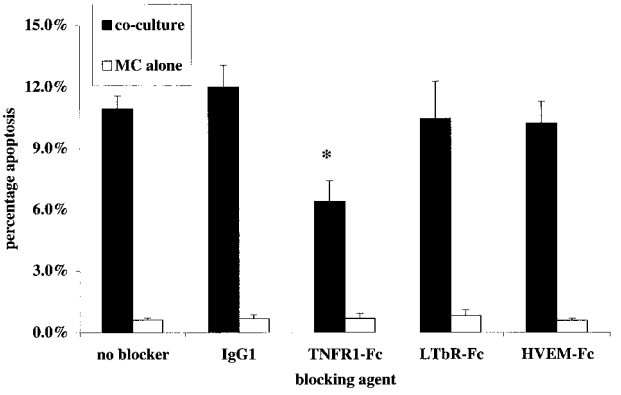

Figure 4. Soluble TNFR1 inhibits nitric oxide-independent rodent Mϕ killing of primed rodent mesangial cells. Murine bone marrow-derived Mϕ were co-cultured with mesangial cells as described in Materials and Methods. Experiments were activated with IFN-γ and LPS as described, with L-NIL (30 μmol/L) to block NOS 2-mediated killing, and soluble Fc-fusion proteins TNFR1-Fc, LTβR-Fc, HVEM-Fc, or isotype control IgG_1 were added in culture medium at 10 μg/ml. Control wells containing mesangial cells but no Mϕ received all reagents. Mesangial cell apoptosis was assessed at 24 hours. Note a reduction in the capacity of Mϕ to induce apoptosis in the presence of TNFR1-Fc. *, $P < 0.05$.

with IgG_1 *versus* 3.9 ± 0.7% mesangial cell apoptosis with 50 μg/ml TNFR1-Fc), but the reduction was not complete. Control rat mesangial cells growing alone with 50 μg/ml TNFR1-Fc showed 1.3 ± 0.7% apoptosis at 24 hours. The IC_{50} for this fusion protein was 5.0 ± 1.2 μg/ml (Figure 5).

We were also interested in determining whether TNFR1 blockade might similarly inhibit activated human Mϕ induction of mesangial cell apoptosis, because human Mϕ production of nitric oxide is notoriously difficult to elicit.[9] Indeed, we confirmed that the IFN-γ/LPS activation regimen had no significant stimulatory effect on human monocyte-derived Mϕ production of nitric oxide (data not shown) and that L-NMMA had no effect on activated human Mϕ killing of IFN-γ-primed human mesangial cells (14.7 ± 2.1% apoptosis at 24 hours with 200 μmol/L L-NMMA *versus* 15.7 ± 1.9% under control conditions). In this physiologically nitric oxide-independent human cell system, soluble TNFR1 again demonstrated selective in-

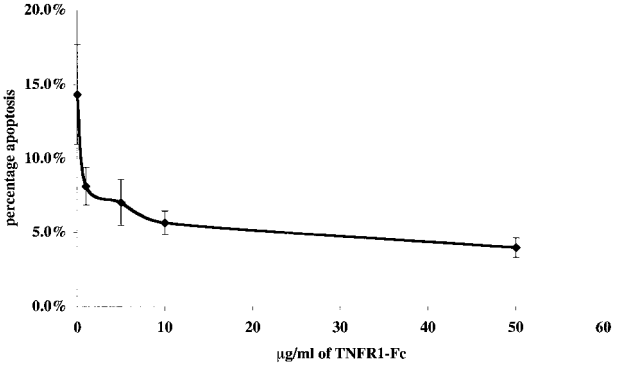

Figure 5. Concentration-dependent inhibition by sTNFR1 of nitric oxide independent rodent Mϕ induction of rodent mesangial cell apoptosis. Using the same co-culture assay of mesangial cell apoptosis, a range of concentrations of TNFR1-Fc was added to wells and apoptosis quantified, compared with 50 μg/ml of IgG_1 control shown as 0 μg/ml of TNFR1-Fc on the curve.

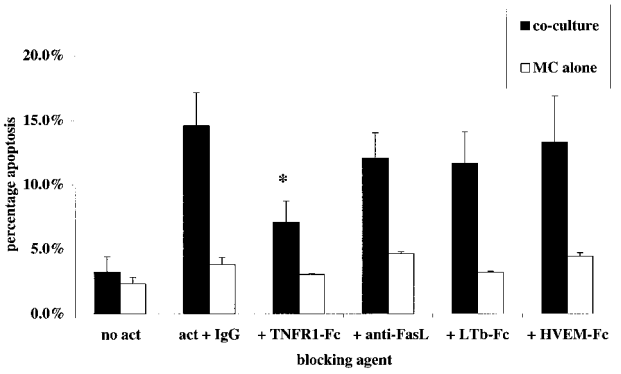

Figure 6. Induction of apoptosis of human mesangial cells by human monocyte-derived Mϕ is mediated by TNFR1 ligation. Co-culture was established using primed human mesangial cells and Mϕ. On activation with IFN-γ plus LPS, soluble Fc-fusion proteins TNFR1-Fc, LTβR-Fc, HVEM-Fc, or isotype control IgG_1 (10 μg/ml) were added in culture medium at 10 μg/ml. Also, the blocking anti-Fas ligand antibody (1 μg/ml) was applied to adjacent wells. Control wells containing human mesangial cells but no Mϕ received all reagents. Note a reduction in the capacity of Mϕ to induce apoptosis in the presence of TNFR1-Fc, but not in the presence of any of the other reagents. *, $P < 0.05$.

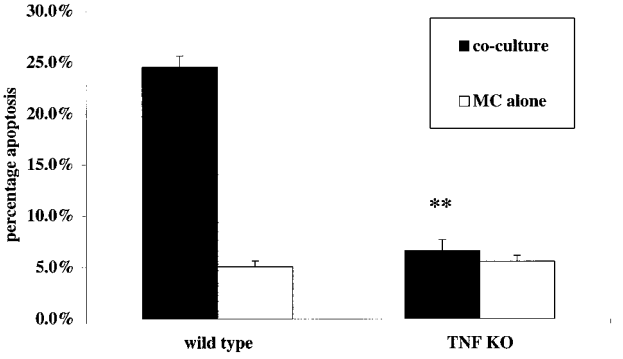

Figure 7. The comparative effect of activated wild-type and *Tnf*α*/Tnf*$\beta$$-/-$ knockout murine Mϕ on apoptosis of mesangial cells primed with IFN-γ. Mesangial cells were primed as described for 24 hours in the presence of IFN-γ (300 U/ml). Co-culture was established using bone marrow-derived Mϕ from either *Tnf*α*/Tnf*$\beta$$-/-$ knockout mice or wild-type littermate controls and activated with IFN-γ (100 U/ml) plus LPS (1 μg/ml) in the presence of L-NIL (30 μmol/L) to block NOS 2-mediated killing. Experiments were assayed after 24 hours. Although the wild-type Mϕ were able to induce mesangial cell apoptosis, the knockout animals incapable of inducing apoptosis greater than that seen in control wells. **, $P < 0.01$ compared with wild-type co-culture ($n = 5$).

hibition of activated Mϕ direction of mesangial cell apoptosis (Figure 6).

Nitric Oxide-Independent Induction of Mesangial Cell Apoptosis Is Restricted by Macrophage TNF

Although the foregoing studies provided strong evidence that ligation of TNFR1 played a major role in nitric oxide-independent Mϕ killing of mesangial cells, the data did not provide direct evidence that Mϕ-derived ligands for TNFR1 (TNF-α and TNF-β) were involved. Furthermore, the failure of sTNFR1 to exert complete inhibition of killing might have reflected its physical exclusion from regions of close contact between macrophages and mesangial cells. To examine this question definitively, we prepared bone marrow-derived (BMD) Mϕ from double-knockout *Tnf*$\alpha$$-/-$, *Tnf*$\beta$$-/-$ mice, and wild-type littermate controls; all animals used were C57/BL6 $\times$ 129sv to ensure that strain differences did not complicate interpretation.[13] Preliminary studies showed both knockout and wild-type Mϕ produced similar amounts of nitric oxide in response to IFN-γ with LPS (50 $\pm$ 4.6 [wild type] *versus* 47.0 $\pm$ 3.3 [*Tnf*α*/*$\beta$$-/-$] nmol nitrite per 10^6 cells per 24 hours) and that this was almost completely inhibited by the iNOS inhibitor L-NIL at 30 μmol/L (to 3.7 $\pm$ 2.1 and 2.9 $\pm$ 1.9 nmol nitrite per 10^6 cells per 24 hours, respectively). Supernatants from Mϕ activated with IFN-γ and LPS confirmed that the *Tnf*α*/*$\beta$$-/-$ Mϕ were completely unable to produce TNF-α, as assessed by enzyme-linked immunosorbent assay of culture supernatants at 24 hours (wild-type Mϕ 3982 pg/ml *Tnf*α*/*$\beta$$-/-$ Mϕ< 0.00 pg/ml). Rat mesangial cells were primed for 24 hours with IFN-γ then co-cultured with either wild-type Mϕ or *Tnf*α*/*$\beta$$-/-$ Mϕ. The co-cultures were activated with IFN-γ (100 U/ml) and LPS (1 μg/ml) in the presence of L-NIL (30 μmol/L). In these experiments knockout Mϕ were completely unable to induce mesangial cell apoptosis compared with controls of mesangial cells growing alone in the presence of cytokines (Figure 7). Wild-type Mϕ induced mesangial cell apoptosis as expected. These data demonstrate that when nitric oxide synthesis was inhibited, and therefore used as a model of human Mϕ-directed apoptosis, Mϕ production of TNF-α/β accounted for all of the killing effect of rodent Mϕ on mesangial cells.

Discussion

Although it has been well documented that Mϕ can kill tumor cells,[6–8] it has only very recently become apparent that macrophages can also direct tissue remodeling by inducing physiological cell death, in nontransformed resident cells.[4,19,20] Thus we have shown previously that activated Mϕ, whether isolated directly from experimentally inflamed glomeruli or generated by IFN-γ/LPS activation *in vitro* can induce apoptosis in cultured primary mesangial cells.[4] In this report, we demonstrate for the first time that activated Mϕ induction of apoptosis in nontransformed cells can be profoundly inhibited by interaction of Mϕ with apoptotic cells. However, in a rodent system this effect could only be demonstrated when nitric oxide production was inhibited. Nevertheless, such nitric oxide-independent direction of mesangial cell apoptosis was inhibitable by soluble TNF receptor in both a rodent cell culture system and a physiologically nitric oxide-independent human cell culture system. Indeed, a major role for TNF in the rodent system was confirmed by the failure of knockout Mϕ to induce nitric oxide-independent mesangial cell apoptosis. Therefore, our key conclusion is that macrophage-derived TNF may play a major role in directing apoptosis of primary, nontransformed glomerular mesangial cells.

We also regard it as interesting and important that TNF-restricted Mϕ direction of mesangial cell apoptosis

was selectively suppressed by interaction of Mϕ with apoptotic cells but not when healthy cells or latex beads were used as control particles. These data are in keeping with the marked suppressive effects of apoptotic cells on activated rodent bone marrow-derived Mϕ killing of tumor cells, in which the reported data favor a major role for a nitric oxide-independent cell killing mechanism because nitric oxide release was only modestly suppressed.[10] Both the latter study and the current work may have methodological differences from experiments in which nitric oxide-directed parasite killing was suppressed by Mϕ binding of apoptotic cells.[21] Nevertheless, a second key conclusion of our work is that interaction with apoptotic cells can suppress TNF-restricted Mϕ direction of mesangial cell apoptosis.

We propose that further work should examine the likelihood that activated Mϕ deletion of resident cells at inflamed sites may be subject to negative feedback control, in which ingestion of apoptotic cells down-regulates Mϕ capacity to induce apoptosis in resident cells. This new concept suggests additional deleterious consequences should Mϕ clearance of apoptotic cells be defective. Previously it has been proposed[22,23] that reduced Mϕ capacity for clearance of leukocytes and other cells undergoing apoptosis, as now demonstrated in *C1q−/−* knockout mice,[24] threatens tissue injury because of the likelihood that cellular contents escaping from noningested apoptotic cells undergoing secondary necrosis will injure tissues directly and indirectly[23] by stimulating Mϕ release of injurious mediators. Our new data indicate that failure to ingest apoptotic cells might deprive activated Mϕ of a crucial off-signal resulting in undesirably prolonged capacity to direct death of neighboring cells by TNF-restricted mechanisms. Nevertheless, it is likely to be some time before this hypothesis can be tested *in vivo*, because a growing body of evidence argues that redundancy in clearance mechanisms may require that a number are disabled before sustained defects in clearance of apoptotic cells can be demonstrated.[25,26]

Furthermore, future work will also need to address whether Mϕ ingestion of apoptotic cells down-regulates Mϕ membrane TNF-α cell-surface expression, or whether integrin co-factors, necessary for successful transduction of the apoptotic signal, are down-regulated.[5] Preliminary data suggest that membrane-bound rather than soluble TNF-α is responsible for Mϕ-directed apoptosis of mesangial cells (J. Duffield and J. Savill, unpublished data), as reported for Mϕ-directed leukocyte apoptosis.[5]

To conclude, when macrophage release of nitric oxide is minimal, as may be expected in human inflammation, these data demonstrate a major role for TNF in activated Mϕ killing of cytokine-primed mesangial cells by apoptosis. Furthermore, inhibition of the capacity to direct apoptosis consequent on Mϕ binding/ingestion of apoptotic cells reveals a new and potentially important negative feedback control mechanism in remodeling of the inflamed site.

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